PTEN-induced kinase 1 (PINK1) and Parkin: Unlocking a mitochondrial quality control pathway linked to Parkinson's disease

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PTEN-induced kinase 1 (PINK1) and Parkin: Unlocking a mitochondrial quality control pathway linked to Parkinson's disease

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Abstract

Dissection of the function of two Parkinson's disease-linked genes encoding the protein kinase, PTEN-induced kinase 1 (PINK1) and ubiquitin E3 ligase, Parkin, has illuminated a highly conserved mitochondrial quality control pathway found in nearly every cell type including neurons. Mitochondrial damage-induced activation of PINK1 stimulates phosphorylation-dependent activation of Parkin and ubiquitin-dependent elimination of mitochondria by autophagy (mitophagy). Structural, cell biological and neuronal studies are unravelling the key steps of PINK1/Parkin-dependent mitophagy and uncovering new insights into how the pathway is regulated. The emerging role for aberrant immune activation as a driver of dopaminergic neuron degeneration after loss of PINK1 and Parkin poses new exciting questions on cell-autonomous and noncell-autonomous mechanisms of PINK1/Parkin signalling *in vivo*.

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Introduction: PTEN-induced kinase 1/Parkin links to Parkinson's disease pathogenesis

Parkinson's disease (PD) has emerged as the fastest-growing neurodegenerative disorder worldwide characterised by motor symptoms including tremor and nonmotor symptoms such as cognitive decline [1,2]. Pathological studies of postmortem brain tissue of patients with PD reveal characteristic loss of dopamine neurons from the substantia nigra pars compacta region in the brain and accumulation of α-synuclein positive inclusions known as Lewy bodies (LBs) [1]. The role of mitochondrial dysfunction in PD emerged in the 1980s with the discovery that mitochondrial toxins, such as 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine, recapitulate selective nigral degeneration in humans and animals, and biochemical analysis of PD brain tissue revealed defects in mitochondrial respiratory chain enzyme complexes [3]. However, the molecular basis of how mitochondrial dysfunction leads to PD remained unknown till pioneering genetic studies that identified approximately 20 genes and loci (designated PARK) linked to monogenic forms of PD [4]. Autosomal recessive-inherited causal mutations in human PTEN-induced kinase 1 (PINK1) and the RING-IBR-RING E3-ubiquitin (Ub) ligase Parkin (encoded by *PARK6* and *PARK2* genes, respectively) were discovered in patients with early-onset PD [5,6] (Figure 1). Molecular analysis demonstrated these proteins function together in a common mitochondrial signalling pathway in which PINK1 phosphorylates Ub (phospho-ubiquitin) and Parkin. This triggers Parkin activation and Ubiquitin-dependent removal of damaged mitochondria by autophagy (mitophagy). This model correlates very well with clinical data in which PINK1 and patients with Parkin PD have indistinguishable phenotypes with early-onset and sustained responsiveness to L-Dopa therapy and similar pathology with general paucity of LBs [7].

Parallel studies of postmortem brain tissue have demonstrated elevated levels of phospho-ubiquitin in aged and sporadic PD cases compared with controls [8,9]. It has also been reported that Parkin undergoes oxidative modifications that render it more insoluble and that it can sequestrate in LBs, leading to reduced availability of soluble Parkin for native functions including as a redox molecule [10,11]. A specific role for PINK1 and Parkin in α-synuclein-driven PD is also suggested from preclinical models in which α-synuclein-induced mitochondrial pathologies and dopamine neurodegeneration are significantly worsened in either PINK1 or Parkin knockout mice [12,13].

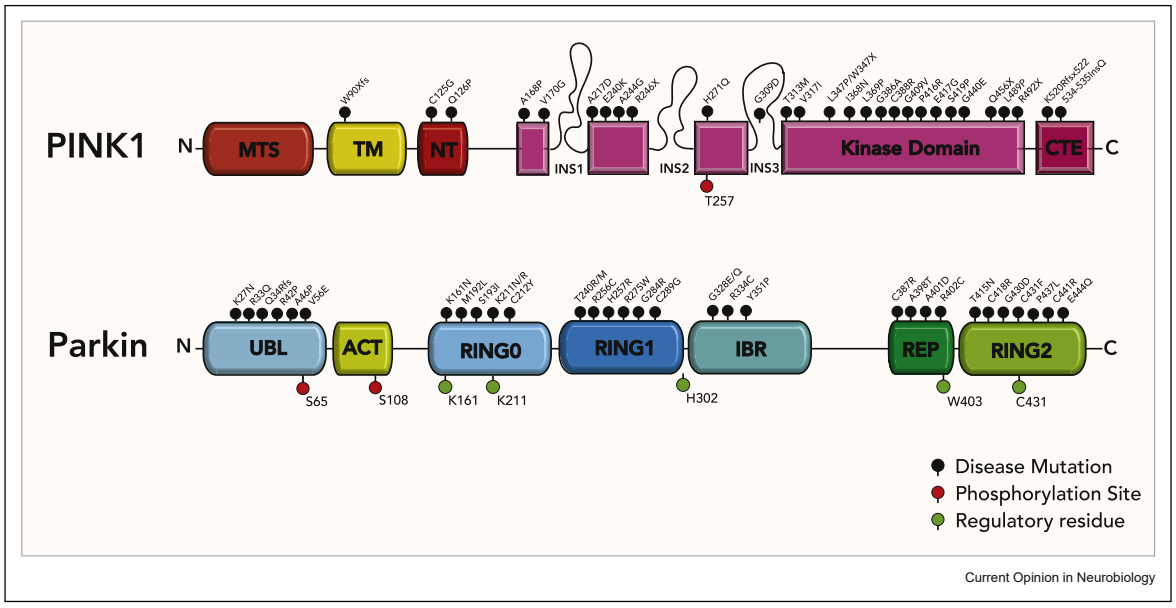
Abbreviations

ACT activating element
AMP adenosine 5-monophosphate
AMPK (AMP)—activated protein kinase
ACC acetyl-CoA carboxylase
ATG9A autophagy-related protein 9A
ATP13A2 ATPase cation transporting 13A2,
AA amino acid
CCCP carbonyl cyanide m-chlorophenyl hydrazone
CRISPR/Cas9 clustered regularly interspaced short palindromic repeats/CRISPR-associated protein 9
CTE C-terminal extension
cGAS cyclic GMP-AMP synthase
CCV clathrin coated vesicle
DA dopamine/dopaminergic
DFCP1 double FYVE-containing protein 1
DUB deubiquitinase
ER endoplasmic reticulum
E2 ubiquitin-conjugating enzyme
fs frame shift mutation
GBA glucocerebrosidase
GMP Guanosine 5-monophosphate
HUWE1 HECT, UBA and WWE domain-containing protein 1
IBR in between RING domain
INS insertion
IMM inner mitochondrial membrane
iPSC induced pluripotent stem cell
IL interleukin
IFN interferon
IFI16 interferon gamma inducible protein 16
KO knockout
LC3 microtubule-associated protein 1A/1B-light chain 3
MPTP 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine
MTS mitochondrial targeting sequence
mesDA mesencephalic dopaminergic
mTOR mechanistic target of rapamycin kinase
mtDNA mitochondrial DNA
MDVs mitochondria derived vesicles
MitAP mitochondrial antigen presentation
MUL1 mitochondrial ubiquitin ligase 1
MARCH5 membrane-associated ring finger (C3HC4) 5
MGRN1 mahogunin ring finger-1
NT N-terminal, regulatory domain
NDP52/CALCOCO2 coiled—coiled domain-containing protein 2
nM nano molar
NF- κB nuclear factor kappa-light-chain-enhancer of activated B cells
NLRP3 NLR family pyrin domain containing 3
OMM outer mitochondrial membrane
OPTN optineurin
PINK1 PTEN-induced kinase 1
PD Parkinson's disease
p-Ub phospho-ubiquitin
Phc Pediculus humanus corporis
PM plasma membrane
RING really interesting new gene domain
REP repressor element
Rab ras-associated binding
SNX sorting nexin family
SN substantia nigra
STING stimulator of interferon genes
Tc Tribolium castaneum
TM transmembrane domain
TOM translocase of outer membrane
TBK1 TANK Binding Kinase 1
TAX1BP1 Tax1-binding protein1
TLR-9 toll-like receptor9
TCR T-cell receptor
UBL ubiquitin-like domain
Ub ubiquitin
ULK1 Unc-51 Autophagy activating kinase 1
USP30 ubiquitin-specific protease 30
WIPI1 WD repeat phosphoinositide-interacting protein 1

Structural view of PTEN-induced kinase 1 and Parkin and impact of Parkinson's disease-causing mutations

Human PINK1 encodes a 581 amino acid (AA) serine (Ser)/threonine (Thr) protein kinase containing an N-terminal mitochondrial targeting sequence, a catalytic kinase domain with three insertions and a C-terminal hydrophobic extension (Figure 1). Most PD-causing mutations lie within the kinase domain highlighting the importance of this region in its protective role against PD (Figure 1). Structural and biochemical analysis of constitutively active insect orthologues of PINK1 namely, *Tribolium castaneum* [Protein Data Bank (PDB): 5OAT] and *Pediculus humanus corporis* in complex with Ub via a nanobody [PDB: 6EQI] has identified a critical role of autophosphorylation of a highly conserved Ser residue (Tribolium castaneum PINK1 (TcPINK1), Ser205; Pediculus humanus corporis (PhcPINK1) Ser202) within the N-lobe for activation and Ub substrate recognition [14,15]. Structural analysis also revealed a previously unidentified role for the third loop insertion (INS3) in Ub substrate recognition and catalytic activity [14,15]. Mutagenesis studies of human PINK1 in cells have confirmed conservation of these mechanisms; however, unequivocal detection of PINK1 autophosphorylation at Ser228 (equivalent to the insect Ser205/202) is outstanding [14,16,17]. The structures have provided major insights into the pathogenic mechanisms of some of the key mutations. Mutations

Figure 1



Domain organisation of human PINK1 and Parkin. Schematic representations of the mitochondrial kinase PINK1 and the RBR-E3 ubiquitin-protein ligase Parkin. Individual domains are colour coded and labelled: MTS, mitochondrial targeting sequence; TM, transmembrane domain; NT, N-terminal, regulatory domain; INS, insertion; CTE, C-terminal extension; UBL, ubiquitin-like domain; ACT, activating element; RING, really interesting new gene domain, IBR, in-between-RING domain; REP, repressor element; fs, frameshift mutation. PINK1, PTEN-induced kinase 1; RBR-E3, RING-IBR-RING-E3 Selected PINK1 and Parkin human disease mutations, the phosphosites and regulatory residues referred in the text are highlighted in the domain structure as black, red and green, respectively.

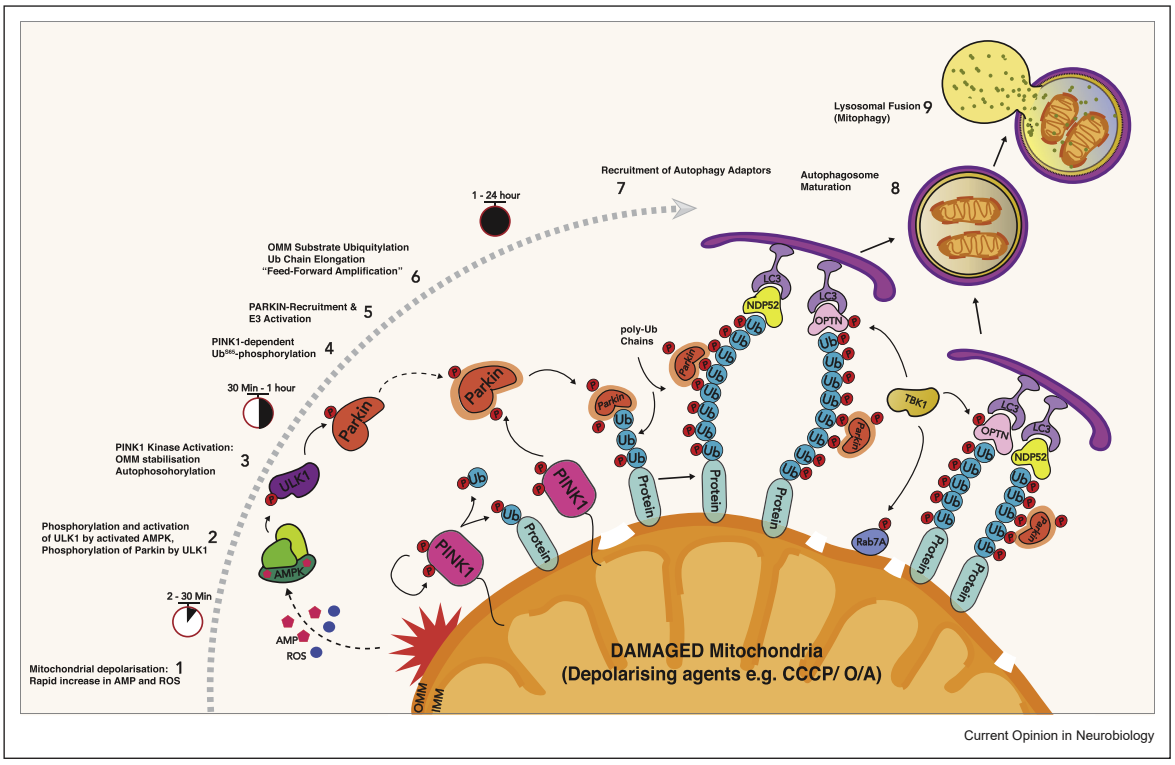
within the ATP-binding pocket (Ala217Asp, Glu240Lys, Ala244Gly and Leu369Pro) and within the activation loop (Gly386Ala, Pro416Arg/Leu and Glu417Gly) both resulted in catalytically inactive PINK1, and mutation within Gly309Asp that lies in INS3 prevented Ub recognition with the remainder of PD mutations being likely to affect the structural integrity of PINK1 (Figure 1) [14,15]. The current insect structures do not provide insights into the role of the N-terminus of PINK1 as well as the mechanism by which PINK1 undergoes dimerisation on activation in cells [18], and understanding these in the structural context of mammalian PINK1 will be essential.

Parkin encodes a 465 AA Ub E3 ligase containing an N-terminal Ub-like (Ubl) domain, RING0, RING1, in-between-RING (IBR), repressor element and RING2 domains. The three RING and IBR domains each coordinate two zinc ions that are essential for protein folding. A previously uncharacterised conserved linker region between the Ubl domain and RING0 termed the activating element (ACT) (AA residues 101–109) has also been identified [19], and it has recently been reported that phosphorylation of a conserved Ser 108 residue within the ACT element by Unc-51 autophagy-activating kinase 1(ULK1) is required for optimal activation of Parkin in cells, and this presages PINK1-mediated phosphorylation of Ser65 within the Ubl domain (Figure 1) [20]. Other key functional residues of Parkin include the catalytic cysteine residue (Cys431) that lies within the RING2 domain [21,22] and His302 on the RING1 domain that has been identified as an essential phospho-ubiquitin-binding residue [23–25]. Numerous PD-causing mutations have been identified including rearrangements that drastically destabilise the protein and missense mutations across the whole protein that disrupt critical regulatory AA residues, for example [Cys431Phe] within RING2 that disrupts the Ub thioester formation required for Ub transfer to substrates [21,22], Ser65Asn that prevents phosphorylation and activation by PINK1 [26], and Lys161Asn Lys211Asn mutants that disrupt the phosphoacceptor pocket on RING0 for pSer65-Ubl thereby preventing release of RING2 and Parkin activation and associated with defective recruitment to damaged mitochondria in cells [19,27].

Upstream mechanism of stress-evoked mitophagy: regulation of Parkin activation by PTEN-induced kinase 1

Under basal conditions, PINK1 is present at low levels owing to proteolytic turnover, and Parkin is localised in the cytosol where it exists in an autoinhibited conformation mediated by three autoinhibitory interfaces [28]. On mitochondrial depolarisation, induced by uncoupling agents (e.g. carbonyl cyanide m-

Figure 2



Model for PINK1-Parkin activation and mitophagy. PINK1-Parkin directed mitochondrial clearance is well-established, and recent findings have further added new paradigms to the molecular regulation of mammalian mitophagy. Mitochondrial damage induced by the depolarising agent such as CCCP/ oligomycin-antimycin causes rapid increase in AMP and mitochondrial reactive oxygen species (mtROS) which is sensed by AMPK within minutes to phosphorylate and activate the kinase ULK1. Activated ULK1 phosphorylates Parkin in its ACT domain marking the first step in mitophagy cascade. Meanwhile, PINK1 also gets autophosphorylated and accumulates on the outer mitochondrial membrane (OMM) where it phosphorylates Parkin to finally activate its E3 ligase activity. Activated Parkin then triggers cycles of ubiquitylation on the OMM, resulting in recruitment of autophagy receptors, autophagosome formation eventually culminating in elimination of damaged mitochondria through mitophagy. AMPK, AMP-activated protein kinase; CCCP, carbonyl cyanide m-chrolophenyl hydrazone; PINK1, PTEN-induced kinase 1; ULK1, Unc-51 autophagy activating kinase 1.

chrolophenyl hydrazone/oligomycin-antimycin), PINK1 is stabilised at the outer mitochondrial membrane (OMM) in association with components of the translocase of the outer membrane complex where it undergoes dimerisation, autophosphorylation and activation (Figure 2) [16,18,29,30]. PINK1 initially phosphorylates Ub attached to OMM proteins specifically at residue Ser65 (phospho-ubiquitin) leading to recruitment of Parkin to the OMM whereon it binds phospho-ubiquitin and undergoes a conformational change that stimulates PINK1 to phosphorylate its Ubl domain at residue Ser65 equivalent to the Ub site (Figure 2) [23–25]. Structural analysis of the regulation of Parkin by PINK1 has revealed the central role of phosphorylation in converting Parkin from an inactive autoinhibited confirmation [31–34] to a fully active enzyme capable of ubiquitylating myriad substrates at the OMM [19,35]. Phospho-ubiquitin binding to Parkin leads to minor movement of the H3 helix (within the RING0 domain), but this is sufficient to dissociate the Ubl domain from the IBR domain and opens up a new pocket at the H3-IBR interface and the RING1 interface for binding of donor Ub and E2; phosphorylation of the Ubl domain leads to its major displacement and binding to a charged pocket within the RING0 domain that disrupts the RING0-RING2 interface to release RING2 availing the catalytic Cys, Cys431 for transthiolation by the charged E2-Ub [19,35].

A recent study has found that mitochondrial depolarisation-dependent PINK1-Parkin activation also converges with the adenosine 5-monophosphate (AMP)-activated protein kinase (AMPK) signalling pathway [20]. AMPK is rapidly activated after any stress that triggers mitochondrial damage depleting ATP levels and concomitantly elevating AMP. In response to mitochondrial depolarisation, AMPK is fully activated in the cytosol within 2 min and directly phosphorylates its substrates Raptor, acetyl-CoA carboxylase (ACC) and ULK1 [20]. Strikingly, Parkin is phosphorylated by

ULK1 at Ser108 within the ACT element at this early time point of minutes followed by rapid recruitment of ULK1 and AMPK to the mitochondria [20]. This presages PINK1-dependent phosphorylation of Parkin and Ub whose temporal dynamics may be slowed by the requirement of PINK1 stabilisation on the OMM (Figure 2) [20].

Downstream mechanism of stress-evoked mitophagy: ubiquitin mediated recruitment of autophagy receptors and mitoautophagosome formation

Studies in HeLa cells and neurons have indicated that accumulation of Ub and/or phospho-ubiquitin on damaged mitochondria stimulates recruitment of autophagy adaptor receptors including optineurin (OPTN), NDP52/(coiled−coiled domain-containing protein 2) and Tax-1-binding protein (TAX1BP1) [36−38]. CRISPR/Cas9 knockout experiments suggest that NDP52 and OPTN are essential for PINK1/Parkin-dependent mitophagy, and TAXBP1 has a lesser effect [37,38], but how these receptors are recruited to damaged mitochondria remains to be fully elucidated. Previous mass spectrometry analysis of ubiquitylation in HeLa cells over-expressing Parkin revealed upregulation of diverse Ub chain types including K6, K11, K48 and K63 [39−42] of which K6 chains were quantified as the predominant chain type [42]. In contrast, in physiologically more relevant cell types that express endogenous levels of Parkin including human embryonic stem cell (ESC)-derived dopamine neurons, human iNeurons and mouse primary cortical neurons, K63 chains were exclusively or predominantly upregulated after mitochondrial depolarisation [39,43]. On PINK1/Parkin activation, OPTN1 recruitment to mitochondria is associated with activation of TANK-binding kinase 1 (TBK1) that phosphorylates OPTN1 to increase its affinity for Ub chain binding that promotes further OPTN1 recruitment to damaged mitochondria and TBK1 activation and also enhances binding to microtubule-associated protein 1A/1B-light chain 3A (LC3 A) to drive mitophagy [37,44]. TBK1 also phosphorylates ras-associated binding (Rab)7 at Ser72 that has been reported to be critical for mitophagy via recruitment of autophagy-related protein 9A-positive vesicles to damaged mitochondria [37]. ULK1, double FYVE-containing protein 1 and WD repeat phosphoinositide-interacting protein 1 stimulate LC3 recruitment and subsequent mitoautophagosome formation. Furthermore, it has been reported that NDP52 and TBK1 jointly recruit a second wave of the ULK1 complex to ubiquitylated cargo at the mitochondria, leading to ULK1 kinase activation, which occurs independently of energy-sensing pathways (AMPK and mechanistic target of rapamycin kinase) and Parkin Ser108 phosphorylation [45]. Consistent with this, reconstitution studies have recently demonstrated that cargo-loaded NDP52, OPTN and TAX1BP1 stimulate LC3 lipidation in the presence of purified autophagy initiation complexes expressed at appropriate physiological (nM) concentrations and furthermore that NDP52 and TAX1BP1 but not OPTN promote ULK1 complex recruitment to the membrane [46]. Recently, it has been proposed that Parkin generates short chains including branched Ub species in both HeLa cells and human iNeurons [42,47], and in future studies, it will be interesting to directly assess the role of specific Ub chain linkages (e.g. K6 vs K63) as well as chain length in a reconstitution system.

PTEN-induced kinase 1/parkin mitophagic signalling in neurons

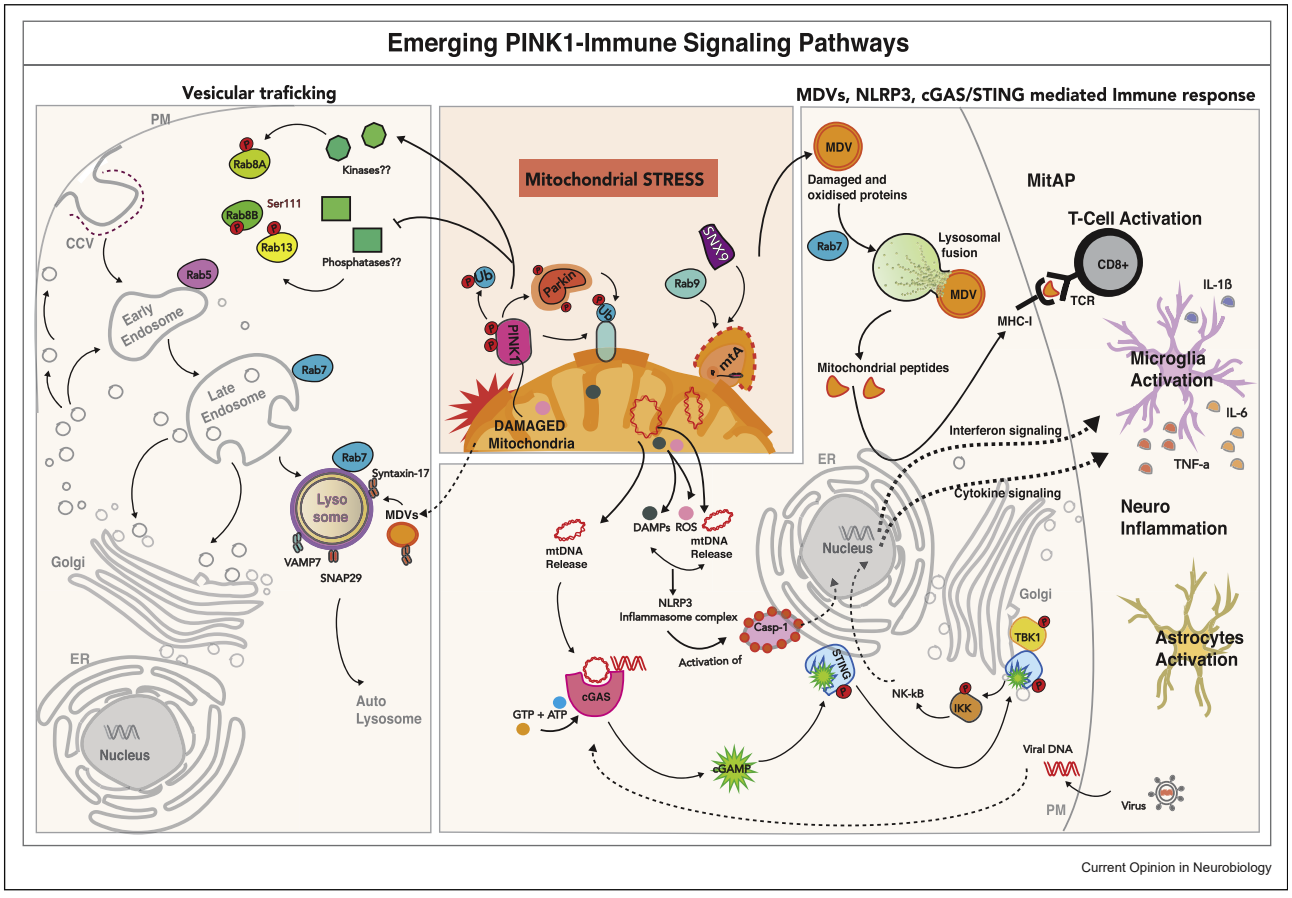
Initial mass spectrometry studies in HeLa cells over-expressing Parkin not also identified myriad substrates, mainly localised to the mitochondria, but also included substrates in the cytosol and other cell compartments [41]. Recent comparative analysis of Lys-ϵ-Gly-Gly (di-GLY) proteomic data sets of human iNeurons and primary mouse cortical neurons in which PINK1/Parkin is expressed under endogenous levels has revealed a common set of 49 diGLY Ub sites spanning 22 OMM proteins [43,47]. This represents a defined Parkin-dependent ubiquitylation signature of damaged mitochondria, and in future studies, it will be interesting to assess this in Dopaminergic (DAergic) neurons as well as non-central nervous system (CNS) cell types. In human iNeurons, it has been demonstrated that increased Parkin-mediated ubiquitylation of OMM substrates is associated with mitochondrial stress-evoked mitophagy that is absolutely PINK1 dependent. This is distinct from basal levels of mitophagy detected in iNeurons that are independent of PINK1 [47]. Basal mitophagy is also independent of PINK1 and Parkin in knockout models *in vivo* [26,48], although these mice lines are reported to exhibit no robust phenotype or only mild phenotypes without striatonigral degeneration, for example, Parkin knockout mice [49,50]. Analysis of Parkin-dependent mitophagy in mouse hippocampal neurons suggests a high degree of spatiotemporal regulation with Parkin/OPTN and TBK1 recruitment occurring in the cell body and rarely in the axon, suggesting alternate pathways may regulate mitophagy in axons [36]. Complementary *in vivo* analysis of *Parkin* knockout mice crossed with the 'mutator' mouse model, that induces mitochondrial stress owing to mutation in *Polg*, led to age-dependent DA neuron loss and levodopa-responsive motor dysfunction [51]. This confirms that endogenous Parkin has a critical role in protecting nigral neurons against mitochondrial dysfunction/accumulation of mitochondrial DNA (mtDNA) mutations *in vivo* consistent with its functions on neurons *in vitro*.

PTEN-induced kinase 1 and parkin mechanisms of immunology linked to neurodegeneration

Innate immune signalling

Akin to bacterial components, escape of mtDNA to the cytosol can trigger innate immune signalling pathways that trigger toll-like receptor 9-mediated type I interferon (IFN) or nuclear factor kappa-light-chain-enhancer of activated B cell inflammatory responses [52] or activation of the NLR family pyrin domain-containing 3 inflammasomes [53]. In a similar manner, the combination of mitochondrial stress and failure to remove damaged mitochondria by loss of PINK1/Parkin has also been shown to stimulate innate immune responses via activation of cyclic GMP-AMP synthase/stimulator of interferon gene (STING)-mediated type 1 IFN response (Figure 3) [54]. PINK1 or Parkin knockout mice crossed with mutator mice or exposed to exhaustive exercise exhibited high levels of cytokines including interleukin (IL)-6, IL-12, and IFN-beta in the serum [54]. Strikingly genetic inactivation of STING blocked exercise-induced cytokine production and prevented the development of neurodegeneration in the double-mutant Parkin/mutator mice [54]. This contrasts with findings in *Drosophila* in which loss of STING does not affect behavioural and mitochondrial phenotypes of PINK1 and Parkin mutant flies [55]. Clinically, patients with Parkin are also found to have higher serum levels of IL-6 than those of controls [54], and this has been recently confirmed in independent

Figure 3



Emerging PINK1 immune signalling pathways. Our knowledge of the involvement of PINK1 and Parkin in cellular mitophagy has expanded, and several new observations suggest their significant role in innate immunity and inflammation. Independent of classical mitophagy, a physiological role for PINK1-Parkin-dependent generation of MDVs enabling the shuttling of damaged mitochondrial proteins to lysosomes has been reported. Mitochondrial antigen presentation (MitAP) of the mitochondrial antigens on Major Histocompatibility Complex 1 (MHC1) molecules then drives mitochondria-specific CD8+ T-cell activation. In addition to MitAP, PINK1-Parkin has also been shown to mitigate proinflammatory signalling pathways causing neuroinflammation. mtDNA released into the cytosol on mitochondrial damage and permeabilisation triggers cGAS-STING-mediated type I interferon response. The mtDNA and Damage associated molecular patterns (DAMPs) also trigger the NLRP3 inflammasome pathway leading to caspase-1-dependent proinflammatory cytokine signalling and activation of glial cells. The activation of the PINK1 signalling pathway also has been implicated in Rab GTPases-mediated vesicular trafficking and autolysosomal clearance. Several Rab GTPases such as Rab8A, Rab8B and Rab13 are known to get phosphorylated on PINK1 activation at the Ser111 residue via yet unidentified phosphorylation events. Abbreviations: mtA, mitochondrial antigen; mtDNA, mitochondrial DNA; cGAS, cyclic GMP-AMP synthase; STING, stimulation of interferon genes; MDVs, mitochondria-derived vesicles; TCR, T-cell receptor; ER, endoplasmic reticulum; PM, plasma membrane; CCV, clathrin-coated vesicle. PINK1, PTEN-induced kinase 1; Rab, ras-associated binding; Ser111, serine 111.

cohorts of patients with PD harbouring biallelic PINK1 and Parkin mutations [56]. Furthermore, a recent study has also found that cytosolic mtDNA is increased in idiopathic PD brain tissues and in zebrafish models of mutant PINK1, glucocerebrosidase (GBA1) and ATP13A2 that are sensed by IFI16 to induce type I interferon responses [57]. These exciting findings raise many questions regarding whether age-dependent mutated or oxidised mtDNA (that might accrue in PD) are differentially sensed to selective immune machinery and which cell types do these mechanisms occur to trigger nigral DAergic neuronal loss in PD and whether this is cell-autonomous or not.

Adaptive immune signalling

Parallel studies in immortalised cell lines indicate that activation of PINK1/Parkin can induce autophagy-independent mitochondrial turnover including mitochondria-derived vesicles (MDVs) that shuttle cargoes to lysosomes for degradation [58,59]. Mechanistically, PINK1/Parkin-dependent MDVs appear to be distinct to that of mitophagy, occurring independent of Dynamin-related protein 1 (Drp1) and requiring the Soluble NSF attachment proteins (SNAP) REceptor (SNARE) syntaxin-17 to mediate MDV endolysosome fusion [59,60]. Whilst MDV formation is dependent on Parkin catalytic activity, how Ub chains or specific substrates are involved in this process remains unknown. Physiologically, MDVs have been linked to mitochondrial antigen presentation (MitAP), and loss of PINK1 and/or Parkin drives MDV formation in immune cells which facilitates MitAP (Figure 3) [61]. Furthermore, exposure of PINK1-knockout mice to intestinal infection by Gram-negative bacteria led to a MitAP response and the induction of cytotoxic mitochondrial CD8+ T cells in the periphery, triggering dopaminergic pathological deficits in the brain and L-Dopa-responsive motor deficits [62]. This raises exciting follow-up studies on the cell specificity of these mechanisms *in vivo* and which specific substrates of PINK1 and Parkin control MDVs and MitAP. Generation of MDVs and MitAP is dependent on SNX9 and Rab9, and SNX9 levels have been observed to be regulated by Parkin, although it is unknown whether SNX9 is ubiquitylated by Parkin [61]. PINK1 has also been reported to induce phosphorylation of a highly conserved Ser111 residue on a subset of Rab GTPases, including Rab8A, Rab8B and Rab13. The regulation of Rabs by PINK1 appears to be indirect, and it remains unknown whether phosphorylation of these proteins influences MDV formation and trafficking [63].

Concluding remarks

PINK1 and Parkin research progress to date has been exemplar in demonstrating how understanding the normal function of PD genes is a critical first step to elucidate the fundamental mechanisms by which mutations lead to DAergic cell loss. This has revealed the central role mitochondrial homeostasis plays in neuronal integrity and survival in the ageing brain. The discovery that loss of PINK1 and Parkin can induce DAergic dysfunction via immune hyperactivation opens up new avenues for investigation on cell-autonomous and noncell-autonomous mechanisms of PINK1/Parkin signalling *in vivo*. Activated microglia and astrocytes play an important role in neuroinflammation in the CNS, and in future work, it will be critical to determine the molecular role of PINK1 and Parkin in these cell types. Therapeutically, activation of PINK1 and Parkin is a potential strategy to prevent neurodegeneration; however, screening and identification of small molecule activators remain challenging in drug discovery research. Defining the negative regulators of PINK1/Parkin signalling has revealed mitochondrial localised Ub-specific protease 30 as a candidate deubiquitinase [64]. Ub-specific protease 30 promotes efficient import of mitochondrial proteins, and its inhibition can enhance PINK1-catalysed phospho-ubiquitin accumulation after mitochondrial damage [47,65]. Another major question is defining parallel mitophagic pathways that may compensate for loss of PINK1 and Parkin. In neurons, multiple proteins are ubiquitylated in response to mitochondrial damage independent of PINK1 and Parkin; however, the ubiquitin E3 ligases that mediate this are unknown [43,47]. Mitochondrial ubiquitin ligase 1 (MUL1; also known as mitochondrial ubiquitin

Box 1. Outstanding questions in the field:

1. What upstream mitochondrial stress pathways activate PINK1-Parkin signalling *in vivo*?
2. Are triggers of mitochondrial damage in idiopathic PD distinct from PINK1/Parkin mechanisms?
3. What is the structure and activation mechanisms of full-length mammalian PINK1?
4. What is the structural mechanism by which Parkin attaches Ub to substrates?
5. What are the physiological Ub signals that signal phagophore formation?
6. What is cell specificity of the STING pathway, and is dopamine cell loss in PD cell-autonomous or noncell-autonomous?
7. What is the cell specificity of MitAP mechanisms of neurodegeneration?
8. What Ub E3 ligases or kinases compensate for loss of PINK1/Parkin *in vivo*?
9. Are there additional DUBs and phosphatases that negatively regulate PINK1/Parkin signalling in neurons?
10. What is the physiological role of PINK1-mediated Rab phosphorylation?

ligase activator of NF- κB (MULAN) and mitochondrial associated protein ligase (MAPL)) functions with Parkin to regulate degradation of paternal mitochondria from sperm, but the mechanism by which MUL1 induces mitophagy is unknown [66]. Furthermore, mitochondrial fusion factors mitofusin1/2 have been reported to be ubiquitylated by MUL1, Membrane-associated RING finger protein 5 (MARCH5), Mahogunin RING finger protein 1 (MGRN1) and HECT, UBA and WWE domain-containing protein 1 (HUWE1) as well as Parkin under distinct cellular stress, but no studies have systematically addressed their interdependence under mitophagic stress conditions [67]. Looking at the future, these and many other questions (Box 1) remain to be addressed to define the key mitochondrial mechanisms relevant to PD pathogenesis.

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Conflict of interest statement

M.M.K.M. is a member of the Scientific Advisory Board of Mitokinin Inc.

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